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Microbial Formation of Poly(3-hydroxyalkanoates)

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Chapter 9

Fluorescent pseudomonads are able to synthesize poly(3-hydroxyalkanoates) (PHAs) as a carbon and energy reserve material. These polyesters, which are optically active polyesters of 3-hydroxy fatty acids, have attracted considerable interest because of possible application as biodegradable plastics.

PHAs can be synthesized from carbohydrate substrates like glucose and from aliphatic substrates like fatty acids. Bacterial cells capable of PHA synthesis start to accumulate PHA when the culture is growth-limited as a result of lack of an essential nutrient in combination with a carbon source excess. By applying these growth conditions in the laboratory to such a bacterial culture of PHA formation can be induced.

In this thesis the biochemical pathways leading to PHA synthesis are studied, the analysis of PHA by GC and NMR techniques is described in various chapters and these techniques are used for the characterization of conventional and novel PHAs. Fermentation processes for the production of PHAs using long chain fatty acids as substrates are developed and studied.

In chapter 2 the synthesis of PHA from glucose is studied. Originally, fluorescent pseudomonads were thought to be unable to synthesize PHAs from carbohydrate carbon sources, however under conditions of growth limitation in combination with a high (>10 g/l) glucose concentration *P. putida* and other fluorescent pseudomonads can synthesize PHA. This polymer is characterized by a high 3-

hydroxydecanoate content and the presence of the unsaturated monomers 3-hydroxy-5-*cis*-dodecenoate and 3-hydroxy-7-*cis*-tetradecanoate. In contrast with PHAs synthesized from alkanes or alkanoates the composition of the glucose derived PHA is not influenced by the substrate. The amount of unsaturated monomers can be influenced by varying the growth temperature of the culture, this results in a higher content of unsaturated monomers at low cultivation temperatures.

On the basis of detailed 1 and 2 dimensional NMR studies the structures of the PHA monomers were determined and on the basis of analogy with the structures of the intermediates of the fatty acid biosynthetic pathway it was hypothesized that these intermediates can be converted to PHA monomers. In addition, it was demonstrated that both *P. oleovorans* PHA polymerase genes could restore the capability to accumulate PHA in a *P. putida* KT2442 *pha* mutant from octanoate as well as glucose.

In chapter 3 the hypothesis that fatty acid biosynthesis is involved in the formation of PHA from glucose is studied by using ^{13}C -labeled substrates for the accumulation of PHA. By using specific inhibitors of the β -oxidation and fatty acid biosynthesis, the relation between PHA synthesis and the fatty acid metabolic routes could be further established.

It was found that PHA synthesized from mixture of decanoate and [^{13}C]-decanoate was ^{13}C enriched at the C-1 carbon atom of the 3-hydroxydecanoate monomers. The 3-hydroxy-octanoate monomers of this PHA were not

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enriched which is in line with the hypothesis that the β -oxidation is involved in the synthesis of PHA from fatty acids.

When PHA as synthesized from a mixture of glucose and $[1-^{13}\text{C}]$ -acetate, all monomers showed ^{13}C enrichment of the odd carbon atoms. This was expected on the basis of the hypothesis that the fatty acid biosynthesis generates the intermediates for PHA formation from carbohydrate substrates. PHA that was synthesized from $[1-^{13}\text{C}]$ -hexanoate provided evidence for the synthesis of PHA monomers via elongation of intermediates presumably with an acetyl-CoA molecule. This was concluded from the labeling pattern of the 3-hydroxy-octanoate monomers. It was also concluded from the labeling pattern that in this case PHA was synthesized via both the β -oxidation and the fatty acid biosynthesis which shows that both pathways can operate in the synthesis of PHA monomers simultaneously. The experiments with acrylic acid and cerulenin, inhibitors of the β -oxidation and the fatty acid biosynthesis respectively, showed that both routes can also function independently in the generation of PHA monomers.

For the structural analysis of PHAs 1 and 2 dimensional NMR techniques are powerful tools. In chapter 4 homo and heteronuclear 2D NMR analysis is used for the structural analysis of PHAs synthesized from linoleic acid and vernolic acid. The latter is a plant derived fatty acid containing an epoxy group in the acyl chain. PHA produced from this substrate contains monomers with epoxy groups at positions that can be predicted from the metabolism of this

fatty acid via the β -oxidation. PHA synthesized from linoleic acid contains unsaturated bonds and the positions of the double bonds can be determined with NMR analysis. In this chapter it is also demonstrated that the fatty acid metabolic route for the degradation of linoleic acid can be deduced from the monomeric composition of the PHA. In this way the involvement of an enoyl-CoA isomerase and a *trans* 2,4 dienoyl-CoA reductase as auxiliary enzymes in a culture of *P. putida* growing on linoleic acid can be demonstrated.

In chapter 5 the gas-chromatographic analysis of PHAs is examined. PHAs can be analyzed on a gas-chromatograph after conversion of the polymer to a mixture of 3-hydroxy fatty acid methyl esters (FAME). This can be done by dissolving the polymer in chloroform and subsequent acid hydrolysis. It was found that for complete conversion of PHA to 3-hydroxy FAME a 4 h acid hydrolysis at 110 °C was necessary. For the similar analysis of PHB a 2h reaction was sufficient. The linearity and the reproducibility of the assay were determined, from this it was calculated that PHB can be assayed with an accuracy of 0.08 mg PHB and medium chain length PHAs can be measured with a 0.3 mg PHA accuracy.

The possibilities of producing PHAs with *P. putida* using long chain fatty acids as substrate were studied in continuous cultures and in fed-batch fermentations. Experiments with chemostats showed that the amount of PHA accumulated in the cell is inversely related to the growth rate of the culture. At a growth rate of $D = 0.05 \text{ h}^{-1}$ the polymer content of the cells is 45 %

of the cell dry mass, at higher growth rates this decreases to 15 %. The composition of the PHA was found to be influenced by the growth rate of the culture, at high growth rates ($D = 0.2 \text{ h}^{-1}$) relatively more 3-hydroxyhexanoate and 3-hydroxyoctanoate monomers were detected than at low growth rates. Interestingly, the molecular weight of the PHA was not influenced by the growth rate.

At optimal cultivation conditions i.e. $D = 0.1 \text{ h}^{-1}$, $C/N = 20$, a maximum volumetric productivity of $0.13 \text{ g PHA / l / h}$ was measured. The volumetric productivity was increased by increasing the biomass concentration in the chemostat. In high cell density continuous culture 30 g / l biomass was maintained in steady-state with 23 % PHA. This corresponds to a productivity of 0.7 g / l / h .

In fed-batch fermentations, *P. putida* was cultivated to high cell densities (92 g / l) with oleic acid as a substrate. Also in this case it was found that the monomeric composition of the PHA changed during the course of the fermentation, this could be explained as a growth rate effect. The molecular weight of the polymer remained constant throughout the fermentation. The highest PHA concentrations were observed at the end of the fermentation when the cells contained 40 % PHA. With this process an overall volumetric productivity of $1.6 \text{ g PHA / l / h}$ could be achieved. The use of long chain fatty acids in fermentation as a substrate for PHA production by *P. putida* has some interesting advantages. The natural variety of fatty acids with respect to the composition of the acyl chain broadens the range of PHAs that can be

produced, especially because the monomeric composition of the PHA can be predicted from the substrate composition. The long chain fatty acids used in these studies can be added to the fermentor as a pure liquid thereby reducing the extra tankfill during fermentation which can be considerable when using water dissolved substrates. Because long chain fatty acids are less oxidized than for example glucose, more oxygen is needed for complete conversion of these substrates to biomass and PHA. This could be disadvantage when producing PHAs on a large scale.

In this thesis the variety of PHAs that can be synthesized by *P. putida* is studied, the biochemical pathways leading to PHA formation are explored and techniques for the analysis of the produced PHA are demonstrated. With the development of fermentations for the production of PHAs from long chain fatty acids the tools for production of a wide variety of PHAs are available. Further optimization of the production of PHA and especially the translation to production on a large scale are the next steps for the development of biodegradable plastics based on poly(3-hydroxyalkanoates).